Peptide nucleic acid–locked nucleic acid polymerase chain reaction clamp-based detection test for gefitinib-refractory T790M epidermal growth factor receptor mutation

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Mutations in the epidermal growth factor receptor (*EGFR*) are observed in a fraction of non-small-cell lung cancers (NSCLS). *EGFR* mutation-positive NSCLS responds to gefitinib. Secondary T790M mutation confers gefitinib resistance to NSCLS. A detection test for the T790M mutation was designed based on the peptide nucleic acid–locked nucleic acid polymerase chain reaction clamp method. The specificity and sensitivity of the test were both greater than 0.99. The test revealed that only a small population of the PC-13 cells carried the T790M mutation. The test also revealed that the T790M mutation was found in none of 151 NSCLC specimens obtained before gefitinib treatment, whereas it was found in four of four specimens obtained from NSCLS that had become refractory to gefitinib. In one patient in whom the L858R-positive *EGFR* allele was amplified to multiple copies, an L858R-T790M double-mutant allele emerged during the gefitinib therapy. This allele was expressed highly. The T790M mutation detection test based on the peptide nucleic acid–locked nucleic acid polymerase chain reaction clamp method is sensitive and specific, and is applicable to clinical practice. It detects T790M-positive cells in the course of gefitinib treatment, and thus will help to devise therapies effective for T790M-positive NSCLS. (*Cancer Sci* 2008; 99: 595–600)

The discovery that mutations in the epidermal growth factor receptor (*EGFR*) gene are found in some patients with non-small-cell lung cancers (NSCLS) and that the presence of these mutations is closely associated with responsiveness to gefitinib (AstraZeneca, London, UK)[1,2] has advanced our knowledge on the carcinogenesis of lung cancers. These gefitinib-sensitive mutations include G719S, G719C, L858R, L861Q, and deletions in exon 19. The mutation-negative lung cancers are typically resistant to gefitinib. Several single-arm, prospective phase II studies in Japan in which gefitinib was given to mutation-positive NSCLC patients have demonstrated a response rate of approximately 70%.[3–6] The frequency of *EGFR* mutations in NSCLS is high in East Asians; more than one-third of adenocarcinomas of the lung carry a mutation in Japan, Korea, and China.[7–9] In Japan, this means that more than 8000 NSCLC patients a year have an *EGFR* mutation, and thus may benefit from gefitinib therapy. The selection of patients with *EGFR* mutation-positive NSCLS is important in clinical practice.

The peptide nucleic acid (PNA)-locked nucleic acid (LNA) polymerase chain reaction (PCR) clamp is a rapid and sensitive method for the detection of *EGFR* mutations.[10] It detects mutations in the presence of a background of 100 to 1000 wild-type sequences. This allows us to identify *EGFR* mutations using a small aliquot from a cytological specimen (sputum, a bronchial washing, a pleural effusion, etc.) that is isolated to establish the diagnosis of NSCLC.[5]

Non-small-cell lung cancers that respond to gefitinib do not always achieve a cure. In time some tumors acquire resistance to gefitinib. The T790M (C2369T) mutation (hereafter T790M mutation) that emerges during the treatment confers gefitinib resistance on the cells.[11,12] Approximately one-half of the cancers that have acquired resistance to gefitinib have been reported to harbor the T790M mutation.[13,14] However, even before gefitinib treatment, a minute fraction of the cancer cells may have a T790M mutation.[15] Gefitinib therapy may select for these cells and cause refractory diseases. Therefore, it may be a better strategy to switch from gefitinib therapy to a different therapeutic regimen when a significant number of T790M-positive cells emerge. This strategy requires a sensitive clinical test to detect the T790M mutation in the presence of a multiple-fold background of wild-type *EGFR* sequence. Again, the PNA-LNA PCR clamp method is suitable to devise such a test.

In the present study, we establish a PNA-LNA PCR clamp-based test for the detection of the T790M mutation. Using this test, we tested the cell lines and clinical specimens. We obtained a pair of NSCLC cells from a single patient before gefitinib therapy and after her disease had become refractory to gefitinib. These cells allowed us to compare the responses to gefitinib, mutations in the *EGFR* alleles, and their expression before and after gefitinib therapy.

Methods

Clinical samples. The present study was approved by the institutional review board of Saitama Medical University and carried out in accordance with the Declaration of Helsinki (1995, revised in Edinburgh 2000). Specimens were obtained from patients who gave their written informed consent. Samples obtained before the initiation of gefitinib treatment included 17 sputums, 63 bronchial washings, three transbronchial biopsies, four needle aspiration biopsies, 17 pleural effusions, one pericardial effusion, 28 paraffin embedded tissues and 18 surgical biopsy specimens, and they were a portion of the samples obtained to establish the

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diagnosis of NSCLC. Some of these samples had been used previously in another study.[5] Specimens isolated from the patients with recurrent disease included three pleural effusions and one bronchial washing.

Cell lines. All cell lines were established from NSCLS. NCI-H1975 (adenocarcinoma) was obtained from the American Type Culture Collection (Rockville, MD, USA). LCSC#1 and 11–18 (adenocarcinomas) were from the Cell Resource Center for Biomedical Research (Tohoku University, Japan). PC-3 (adenocarcinoma) was from the Japanese Collection of Research Bioresources (Tokyo, Japan). LC2/ad, PC-14 (adenocarcinomas), and RERF-LC-A1 (squamous cell carcinoma) were from the Riken Bioresource Center (Tsukuba, Japan). PC-7, PC-9, and PC-13 (adenocarcinomas) were from IBL (Takasaki, Japan). KTSq-1 was provided by Drs Toru Kameya and Shi-Xu Jiang (Kitasato University, Japan).

Cell cultures. The cell lines were expanded in RPMI-1640 medium containing 10% fetal calf serum. The cells in the effusions from patient 1 were cultured in the same medium for several cell divisions to enrich cancer cells.

Cell growth assay. Gefitinib was kindly provided by AstraZeneca. The cells were plated in a 96-well plate (2500–5000 cells/well) and incubated (37°C, 5% CO_2) for 72 h in the presence of 0, 0.001, 0.01, 0.1, 1, and 10 μM gefitinib. The number of viable cells was measured using the Cell Counting Kit (Dojindo Laboratories, Kumamoto, Japan), which utilizes a tetrazolium salt, WST-1. The experiment was done in quadruplicate.

PNA-LNA PCR clamp. Genomic DNA was purified using the QIAamp DNA Micro Kit (Qiagen Japan, Tokyo, Japan). The PNA-LNA PCR clamp reaction was carried out using the Smart Cycler II (Cepheid, Sunnyvale, CA, USA) as described previously.[10,16] Primers were: amplification primers, Ex20-F 5′-CAGAAGCCTACGTGATGG-3′ (forward) and Ex20-B 5′-ACCTTTGCGATCTGCACAC-3′ (backward); a PNA clamp primer, T790Mcl NH_2-CTCATCACGCAGCTCA-COOH; a fluorescence-labeled detection probe, T790Mp 6-FAM/CTCATCA + TGCAGCTCATG/BlackHoleQuencher-1 (a plus sign indicates that the subsequent nucleotide is a locked nucleic acid); and a fluorescence-labeled total probe, Ex20t Cy5/CAGTACCTGCT-CAACTGGTG/BlackHoleQuencher-2. The thermal cycling profile was 95°C for 30 s followed by 45 cycles of 95°C for 3 s and 58°C for 30 s.

Measurement of mRNA expression. The total RNA was isolated and reverse transcribed. The copy numbers of the *EGFR* cDNA that contained the L858R and the T790M mutations were semiquantified using reaction mixture that had the same composition as that used for the PNA-LNA PCR clamp reaction but lacked PNA. The amplification primers were: cDNA-F 5′-CTTACACCCAGTGGAGAAGC-3′ and cDNA-B 5′-CAATGCCATCCACTTGATAGG-3′, which amplify a 576-bp *EGFR* cDNA sequence that spans from exons 18 through 22. The detection probes were T790Mp (see above) and L858Rp.[10] The PCR product was subcloned into the pT7BlueT cloning vector (EMD Chemicals, Darmstadt, Germany) and the nucleotide sequences of the inserts were determined.

Chromogenic in situ hybridization. The chromogenic *in situ* hybridization (CISH), which detects the *EGFR* gene, was carried out using the ZytoDot SPEC EGFR Probe Kit (ZytoVision, Bremerhaven, Germany).

Results

PNA-LNA PCR clamp reaction for detection of the T790M mutation. We first investigated the detection limit of the PNA-LNA PCR clamp reaction for the T790M mutation. We used NCI-H1975, which is heterozygous for the T790M mutation[17] (Fig. 1a), as a positive control and normal human genomic DNA (HG) as a negative control. By diluting NCI-H1975 DNA with

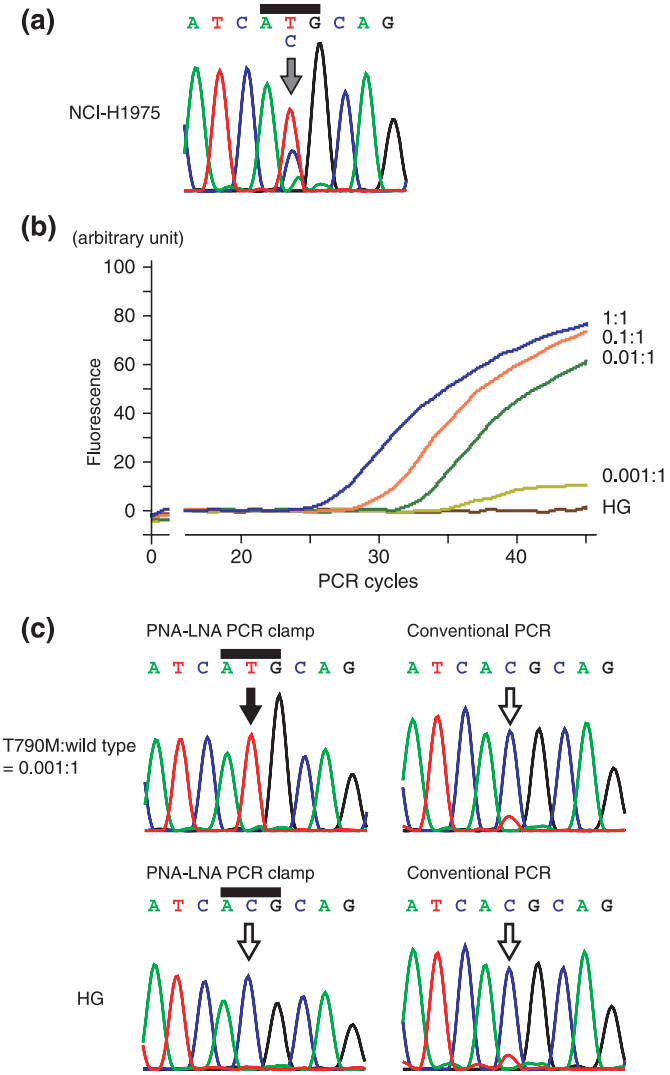

Fig. 1. (a) The direct sequencing chromatogram for NCI-H1975 genomic DNA around codon 790. A bar on the sequence shows the position of codon 790. (b) The peptide nucleic acid (PNA)-locked nucleic acid (LNA) polymerase chain reaction (PCR) clamp reaction for the detection of the T790M mutation. Cocktails that contain both the T790M mutant sequence and the normal epidermal growth factor receptor (*EGFR*) sequence at ratios of 1:1, 0.1:1, 0.01:1, and 0.001:1 were prepared by mixing NCI-H1975 genomic DNA and normal human genomic DNA (HG). The negative control contained normal HG only. (c) Sequencing chromatograms for the PNA-LNA PCR clamp product and for the conventional PCR product. The black arrow indicates the mutated nucleotide, whereas the white arrows indicate the wild-type nucleotide.

HG, we made cocktails of genomic DNA containing the mutant (T790M) and wild-type *EGFR* sequences at ratios of 1:1, 0.1:1, 0.01:1 and 0.001:1. The PNA-LNA PCR clamp reaction detected the mutation in the presence of up to a 1000-fold background of the wild-type *EGFR* sequence (Fig. 1b). In the PNA-LNA PCR clamp reaction, the mutant sequence is amplified preferentially, whereas the wild-type sequence is blocked by the PNA clamp primer.[10] In order to confirm these findings, we subjected the cocktails to both the PNA-LNA PCR clamp and conventional PCR, and sequenced the products directly (Fig. 1c). Even in the presence of a 1000-fold background of wild-type *EGFR*, the PNA-LNA PCR clamp amplified the mutant sequence preferentially, and it was the dominant reaction product (Fig. 1c, upper panels). However, in the absence of the mutant sequence,

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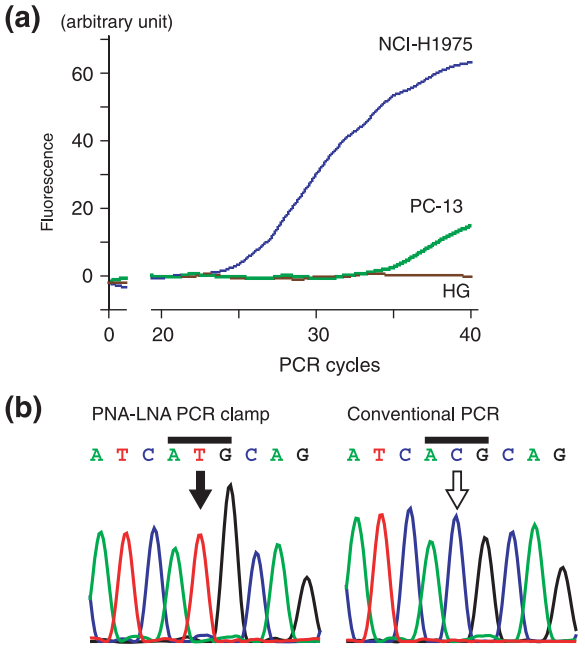

Fig. 2. (a) The peptide nucleic acid (PNA)-locked nucleic acid (LNA) polymerase chain reaction (PCR) clamp amplification curves for PC-13. The curves for NCI-H1975 (positive control) and for normal human genomic DNA (HG; negative control) are shown simultaneously. (b) Sequencing chromatograms from the PNA-LNA PCR clamp product and from the conventional PCR product. The black arrow indicates the mutated nucleotide, whereas the white arrow indicates the wild-type nucleotide.

the wild-type sequence that escaped inhibition by the PNA clamp primer was amplified (Fig. 1c, lower panels).

We next investigated the sensitivity and specificity of the reaction using a 0.01:1 cocktail, because a setup of the reaction that detects the T790M mutation in the presence of a 100-fold wild-type background has been shown to be relevant for clinical use.[16] For positive samples, we prepared 100 cocktails using NCI-H1975 DNA and genomic DNA isolated from 100 different volunteers. For negative samples, we used the same 100 normal DNA without adding the NCI-H1975 DNA. For these 200 reactions (100 positives and 100 negatives), the PNA-LNA PCR clamp presented no false-positive results and no false-negative results (data not shown). We concluded that the sensitivity and specificity of the reaction were both greater than 0.99.

T790M mutation in the cell lines. We next investigated the T790M mutation in NSCLC cell lines derived from the Japanese subjects. The cell lines investigated were PC-3, PC-7, PC-9, PC-13, PC-14, 11–18, LCSC#1, LC-2/Ad, RERF-LC-A1, and KTSq-1. PC-13 (previously demonstrated as wild type for *EGFR*) was the only cell line that had the T790M mutation. The amplification curve was slower to rise than that for NCI-H1975, indicating that only a small population (approximately 1%) of the cells contained the T790M mutation (Fig. 2a). To confirm this, we compared the products of the PNA-LNA PCR clamp reaction and those of the conventional PCR by direct sequencing. As shown in Fig. 2b, the PNA-LNA PCR clamp reaction produced the T790M mutant sequence, whereas conventional PCR produced the wild-type sequence, indicating that only a small population of the PC-13 cells contains the T790M mutation.

T790M mutation in patients. We investigated the presence of the T790M mutation in clinical specimens from 151 NSCLC patients obtained before gefitinib treatment. Among these, four patients provided a pair of samples that was collected before gefitinib treatment and after his/her disease had become refractory to gefitinib (Tables 1,2). None of the 151 samples obtained before a treatment contained the T790M mutation. However, all four samples from the gefitinib-refractory NSCLS contained the T790M mutation in addition to the gefitinib-sensitive mutation that had been detected before treatment. Fig. 3 shows the results for two of these patients following the development of refractory disease. The right panels are the curves for control samples

Table 1. Summary of histology and epidermal growth factor receptor (*EGFR*) mutations

	Before gefitinib treatment	With refractory disease
No. patients	151	4
Histology		
Adenocarcinoma	100	4
Squamous cell carcinoma	25	0
Adenosquamous cell carcinoma	2	
Pleomorphic	1	0
Unclassified	23	0
EGFR mutation status		
Mutation negative	101	0
Gefitinib sensitive†	50	4‡
T790M	0	4‡

†Gefitinib-sensitive mutations detected in the samples include G719S (G2155A), L858R (T2573G), E746-A750del (2235–2249del), E746-A750del (2236–2250del), E746-S752del insV (2237–2254del, C2255T), E746-T751del insV (2237–2251, C2252T), L747-E749del A750P (2239–2247del, G2248C), L747-S752del E746V (2238–2255del, A2237T), L747-S752del P753S (2240–2257del), L747-T753del insA (2239–2258del, insGCT), L747-P753del insQ (2239–2258del, insC), E746-S752del insD (2239–2258del, insCA), and L747-T751del (2238–2252del). ‡All patients had adenocarcinoma, where both T790M and gefitinib-sensitive mutations (L858R, T2573, two cases), E746-A750del (2235–2249del, one case), and E746-A750del (2236–2250del, one case) were detected simultaneously.

Table 2. Characteristics of patients with T790M mutations

Patient	1	2	3	4
Stage	T4N3M1	T1N1M1	T2N0M1	T4N1M1
Histology	Adenocarcinoma	Adenocarcinoma	Adenocarcinoma	Adenocarcinoma
Chemotherapy prior to gefitinib	GEM CDDP + VNR	CBDCA + DTX	DTX	GEM + NAV CBDCA + DTX
Duration of gefitinib therapy (months)	7	10	19	16
Site of relapse	Pericarditis	Bone metastasis	Pleural effusion	Pulmonary metastasis
Chemotherapy after gefiitnib (response)	None	None	DTX (NR)	CDDP + TS-1 (SD)
Overall survival after diagnosis (months)	29	16	25	36 <

CBDCA, carboplatin; CDDP, cisplatin; DTX, docetaxel hydrate; GEM, gemcitabine hydrochloride; NR, no response; SD, stable disease; TS-1, tegafur gimeracil oteracil potassium; VNR, vinorelbine ditartrate.

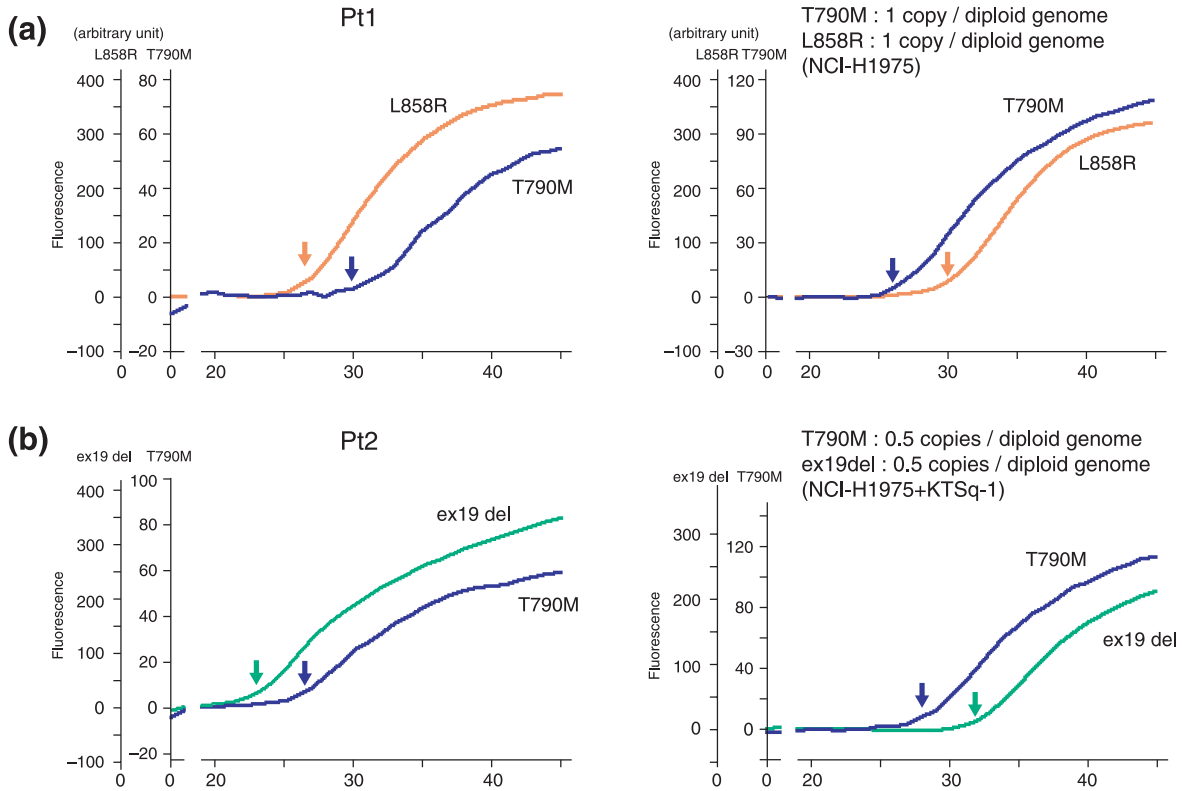

Fig. 3. T790M mutation observed in patients. (a) The amplification curves for patient 1. (b) The amplification curves for patient 2. Right panels show the curves for control samples that contain (a) one copy of both T790M and L858R or (b) 0.5 copies of both T790M and a deletion in exon 19 (E746-A750del) per diploid genome. The colored arrows indicate the positions of the peaks for the second derivatives of the amplification curves.

that contained the T790M and gefitinib-sensitive mutations at 1 (Fig. 3a, right panel) and 0.5 (Fig. 3b, right panel) copies per diploid genome, respectively. The arrows indicate the positions of the peak of the second derivative of each amplification curve calculated by the Smart Cycler software. The cycles that give a peak are known to correlate with the copy numbers of the target molecules. In both patients, multiple copies of the L858R-positive allele or the exon 19 deletion-positive allele existed per single copy of the T790M-positive allele.

Detailed analysis of patient 1. The samples from patient 1 were composed largely of cancer cells, and contaminating normal cells were removed easily by a short-term cultivation. This allowed a detailed analysis of the cancer cells from patient 1.

The cells from the refractory disease were more resistant to gefitinib (concentration that inhibits cell growth by 50% [IC_{50}] = 2 μM) than the cells before the treatment (IC_{50} = 0.06 μM), although they were both more sensitive than NCI-H1975 (IC_{50} > 10 μM) (Fig. 4a). Fig. 4b shows the amplification curve for the sample obtained before the treatment, where a triangle indicates the expected position of the peak of the second derivative when the target molecule existed at a single copy per diploid genome (see Fig. 3a, right panel) and an arrow indicates the actual peak. The distance between the triangle and the arrow indicated that the L858R-positive allele was amplified to approximately 100 copies per cell. Accordingly, the direct sequencing result of the conventional PCR product presented the L858R mutant sequence, and the wild-type sequence was scarcely observed (Fig. 4c). CISH using an *EGFR* gene fragment as a probe revealed two clusters of *EGFR* alleles in the nucleus, confirming the amplification of the *EGFR* alleles (Fig. 4d). In the sample obtained from the refractory disease, the amplification of the L858R-positive allele remained unchanged, whereas the T790M mutation emerged (Fig. 4e). In Fig. 4e, a triangle (the position of the expected peak for a single copy of the target molecule per diploid genome) is located close to an arrow (the actual peak), indicating that the copy number of the T790M-positive allele is approximately one copy per diploid genome (i.e. a single copy per cell).

The results shown above indicated that even in the presence of multiple copies of the L858R-positive allele, a small number of the T790M-positive alleles conferred gefitinib resistance on the cells. We examined mRNA expression using the cells from the refractory disease. Here, a cDNA fragment containing both codons 790 and 858 were amplified by conventional reverse transcription (RT)-PCR, and the numbers of T790M and L858R sequences were compared. The positions of the arrows indicate that the T790M and the L858R sequences existed at a ratio of 1:10 (Fig. 4f). Next we subcloned the RT-PCR product into a plasmid, picked up 13 clones and sequenced them. Two out of the 13 clones had both the T790M and L858R mutations; the remaining 11 had only the L858R mutation; and no cloned sequences were wild type for both codons 790 and 858. This indicated that T790M emerged on an L858R-positive allele and produced a T790M-L858R double-mutant allele. This also indicated that the ratio of the copy numbers of T790M-positive cDNA and the L858R-positive cDNA was approximately 1:10. As presented above, the T790M-positive allele and the L858R-positive allele existed at a ratio of 1:100 (Fig. 4e). These results indicate that the T790M-L858R double-mutant allele was expressed highly, which may contribute to the acquired resistance to gefitinib.

Discussion

In the present study, we developed a PNA-LNA PCR clamp-based test for detection of the T790M mutation and applied it to

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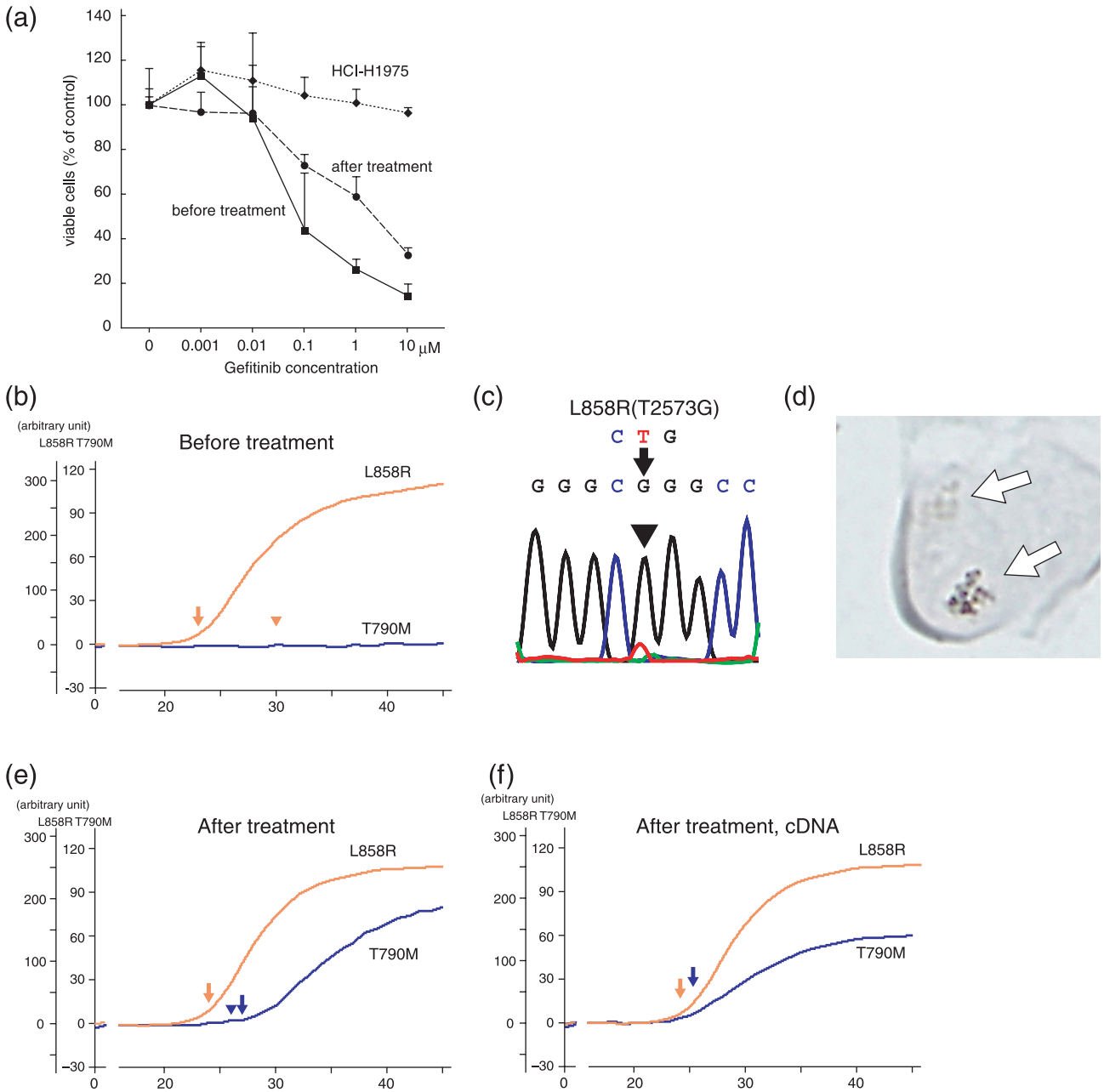

Fig. 4. Detailed analyses of the sample from patient 1. (a) The effect of gefitinib on the cells *in vitro*. Error bars represent standard deviation. (b) The amplification curves for the samples obtained before the treatment. A triangle indicates the expected position of the peak of the second derivative of the curve for the L858R mutation if it exists at one copy per diploid genome. An arrow indicates the actual position. (c) Sequencing chromatogram for the conventional polymerase chain reaction product. (d) Chromogenic *in situ* hybridization result. Arrows indicate the two clusters of the epidermal growth factor receptor (*EGFR*) gene observed in a single nucleus. (e) The amplification curves for the samples obtained after the disease had become refractory to gefitinib. A triangle indicates the expected position of the peak of the second derivative of the curve for the T790M mutation if it exists at one copy per diploid genome. An arrow indicates the actual position. (f) The amplification curves for cDNA.

the clinical samples. The PNA-LNA PCR clamp-based test for gefitinib-sensitive mutations uses five reactions that run in parallel.[10] The reaction for the T790M mutation was added as the sixth reaction. These six reactions are running as a routine clinical test in our university.

To detect the T790M mutation in clinical samples, several issues have to be taken into consideration. First, clinical samples contain many normal cells. Second, only a subset of NSCLC cells may contain the T790M mutation, as shown in the PC-13 cell line. Third, only a subset of the *EGFR* alleles in the cancer cells may contain the T790M mutation, as shown in patients 1 and 2 and as reported in the literature.[18] All of these issues make detection of the T790M mutation difficult because each may obscure the T790M mutation by increasing the background with wild-type sequences. This may lead to a false-negative result and to an underestimation of the role of T790M in acquired resistance to gefitinib. The PNA-LNA PCR clamp method is able to detect the mutation in the presence of a 100- to a 1000-fold background of the wild-type sequence, and therefore is suitable in the clinical setting.

We could not detect the T790M mutation in 151 pretreatment samples. This is consistent with a previous report,[13] and indicates that the number of cells carrying the T790M mutation in pretreatment NSCLS is very small, if it exists at all. In contrast, four of four gefitinib-refractory NSCLS contained the T790M mutation, suggesting that the T790M mutation is one of the major mechanisms in the development of gefitinib-refractory tumors. Previous reports have shown that the T790M mutation is found in approximately half of gefitinib-refractory patients.[13,14] However, the rate might be increased if a more sensitive detection method had been used. Using conventional PCR direct sequencing for detection of the T790M mutation, we may not have detected it in either the PC-13 cell line or samples from patients 1 and 2 because the wild-type sequence existed in large excess. Our method will help to elucidate the overall contribution of T790M in acquired resistance to gefitinib.

Lung cancers frequently recur as blood-borne metastases that are hard to isolate in specimens without invasive procedures. Therapeutic regimens specifically effective for T790M-positive cancers have not yet been established. This limits the isolation of specimens using invasive procedures, and, in the current study, we could only investigate recurrent cancers from which specimens were obtained safely. However, in clinical studies that develop therapies effective for T790M-positive cancers, invasive procedures will be accepted. Our method will help to conduct such studies because it is highly sensitive and examines a variety of specimens.

The T790M mutation produces a bulky methionine side chain that sterically inhibits the binding of gefitinib to the ATP-binding pocket of EGFR.[11] In addition, recent reports have shown that the T790M mutation increases the kinase activity of the *EGFR* gene with gefitinib-sensitive mutations. Therefore, the T790M mutation not only abrogates the effect of gefitinib but also makes the EGFR protein more oncogenic.[19,20] These results suggest that cells that highly express the T790M-positive allele have a higher growth advantage over cells that express the T790M allele at a lower level and thus are selected in the presence of gefitinib. This may be the reason why the T790M-positive allele was expressed highly in patient 1 after the treatment.

Malignant tumors consist of cells with heterogeneity. Heterogeneity is one of the main causes of the development of acquired drug resistance, and thus needs to be taken into consideration when devising therapeutic regimens. The T790M mutation provides an excellent model to study how heterogeneity in a cell population induces resistant tumors. The test we developed in the present study can detect T790M mutant-positive cells with high specificity and sensitivity. This test is not only useful as a clinical test but will provide an insight into the management of cancer heterogeneity in order to achieve successful treatment.

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